ELEVATED IMMUNOREACTIVE TUMOR NECROSIS FACTOR AND INTERLEUKIN-1 IN SICKLE CELL DISEASE

Robert B. Francis, Jr, MD, and L. Julian Haywood, MD
Los Angeles, California

To determine whether tumor necrosis factor (TNF) and interleukin-1 (IL-1) might be involved in the pathogenesis of sickle cell disease and its complications, TNF-alpha and IL-1-alpha were measured using enzyme-linked immunosorbent assay in 59 plasma samples from 34 adult subjects with Hb SS or Hb SC who did not have documented infections. Tumor necrosis factor was elevated on at least one occasion in 27 subjects, including 18 of 21 subjects in the steady state and 13 of 19 subjects during painful crisis. Interleukin-1 was elevated on at least one occasion in 6 subjects, including 3 subjects in the steady state and 3 subjects in crisis. All subjects with elevated IL-1 also had elevated TNF. Tumor necrosis factor and IL-1 were similarly elevated in the steady state and during painful crisis. No correlation was noted between TNF or IL-1 levels and the extent of activation of coagulation, as measured by plasma levels of the fibrin D-dimer fragment, the overall severity of vascular occlusive disease in each subject, or the presence of specific vascular occlusive complications. We conclude that plasma TNF is frequently elevated in subjects with sickle cell disease, and IL-1 is also elevated in some subjects. A direct role for these cytokines in the pathogenesis of vascular occlusion in sickle cell disease was not demonstrated, but an indirect role was not excluded. (*J Natl Med Assoc.* 1992;84:611-615.)

Key words • sickle cell disease • tumor necrosis factor • interleukin-1 • cytokines

Most of the morbidity and mortality in sickle cell disease is caused by either infection or vascular occlusion.[1,2] Infection is primarily a problem in younger children with sickle cell disease, whereas older children and adults suffer more from vaso-occlusive complications.[1,2] The pathogenesis of vascular occlusion in sickle cell disease is still incompletely understood, but it appears to involve the abnormal adhesive, rheologic, and procoagulant properties of the sickle erythrocyte.[3,4]

Tumor necrosis factor (TNF) and interleukin-1 (IL-1) are endogenous cytokines that exert profound, pleiotropic effects on metabolism, the immune system, endothelial function, and hemostasis.[5-8] Endotoxin is an extremely potent stimulus of TNF and IL-1 secretion by macrophages and other cells.[9-11] Recently, it was reported that some individuals with sickle cell disease have circulating endotoxin in the absence of symptomatic infection, possibly as a result of reticulendothelial dysfunction leading to impaired clearance of endotoxin absorbed from the gut.[12] This suggests that TNF and IL-1 might be elevated in some subjects with sickle cell disease and could contribute to the pathogenesis of vascular occlusion. To further investigate this possibility, TNF and IL-1 were measured using enzyme-linked immunosorbent assay (ELISA) in a large number of subjects with sickle cell disease, both in the steady state and during episodes of painful crisis.

From the Department of Medicine, University of Southern California School of Medicine, Los Angeles, California. This work was supported by a grant from the Sickle Cell Disease Research Foundation of Los Angeles. Requests for reprints should be addressed to Dr Robert B. Francis, Jr, USC School of Medicine, Raulston Bldg Rm 304, 2025 Zonal Ave, Los Angeles, CA 90033.

MATERIALS AND METHODS

Study Subjects and Blood Collection

Adult subjects with Hb SS, Hb SC, or Hb S-beta thalassemia were eligible for inclusion in the study provided they did not have either a documented infection or a blood transfusion within the previous 3 months. Subjects were considered to be in vaso-occlusive crisis if they reported the recent onset of acute musculoskeletal pain without antecedent trauma or infection. Subjects were considered to be in the steady state if such a history was lacking.

After written informed consent was obtained, each subject completed a standard questionnaire regarding past and current clinical complications and frequency of painful crises, from which an overall vascular occlusive score was calculated as described by Hebbel et al.[13] Subjects also subjectively quantitated both their current and usual level of pain on a scale of 0 to 100, with 0 representing no pain and 100 representing the worst pain they had ever experienced.

Blood was collected by standard peripheral vein venipuncture into citrate anticoagulant. Plasma was separated by centrifugation for 10 minutes at 12 100 g at 4°C and stored at −80°C until used. As a control, citrated plasma samples were obtained from apparently healthy young adult subjects. No attempt was made to match sickle cell and control subjects for age, race, or sex. These investigations were carried out with the approval of the Human Subjects Committee of the Los Angeles County-University of Southern California Medical Center in accordance with the principles of the Helsinki Declaration of 1975. The results of fibrin D-dimer measurements in these subjects have been reported in more detail elsewhere.[14]

Assay Methods

Plasma TNF-alpha and IL-1-alpha were measured using ELISA (Endogen, Boston, Massachusetts), according to the manufacturer's instructions. Since the 60 pg/mL TNF standard and the 75 pg/mL IL-1 standard were the lowest points on the respective standard curves that consistently gave a reading above background (buffer blank), plasma TNF and IL-1 levels were considered elevated only if they were >60 pg/mL and >75 pg/mL, respectively, and were considered to be undetectable if they were below these levels. Plasma fibrin D-dimer fragment was measured using ELISA (American Diagnostica, Greenwich, Connecticut) as previously described.[14] The previously established normal range in our laboratory for D-dimer is 79 ± 25 (SD) ng/mL.[14] Plasma fibrinogen was measured using the ACA method (Dupont, Wilmington, Delaware) by the Clinical Laboratory of the Los Angeles County-University of Southern California Medical Center.

Statistical Methods

The significance of differences in the mean values of TNF, IL-1, D-dimer, and fibrinogen was assessed using the two-tailed Student's *t* test. Correlation was assessed using simple linear regression. Statistical significance was defined as a *P* value of <.05.

RESULTS

Immunoreactive TNF-alpha and IL-1-alpha were measured in a total of 59 samples from 34 adult subjects with sickle cell disease. The Table lists the TNF, IL-1, D-dimer, and fibrinogen values for all 59 samples. Subjects ages ranged from 19 to 37; there were 11 women and 23 men. No subject was pregnant, taking oral contraceptives, or experiencing hepatic or renal failure. Thirty subjects had Hb SS, and four subjects had Hb SC. Twenty-one subjects (22 samples) were in the steady state; 19 subjects (37 samples) were in acute painful crisis. Six subjects were tested at least once both during acute crisis and in the steady state. Serial samples were obtained on different days of a single episode of painful crisis from 10 subjects. Tumor necrosis factor and IL-1 also were measured in eight healthy young control subjects, ages 23 to 36, three females and five males.

Plasma TNF was elevated on at least one occasion in 18 of the 21 steady-state sickle cell subjects and in 13 of the 19 crisis subjects. Tumor necrosis factor was undetectable in all eight healthy control subjects. Tumor necrosis factor levels ranged from <60 to 780 pg/mL in the steady state and from <60 to 725 pg/mL in crisis. Mean TNF was 150 ± 171 (SD) pg/mL in the steady state and 151 ± 181 pg/mL in crisis. In nine of the 14 subjects from whom more than one sample was obtained, TNF was either consistently elevated or consistently undetectable.

Plasma IL-1 was elevated in three subjects in the steady state and in three subjects in acute crisis, all of whom also had elevated TNF. Of the 14 subjects from whom more than one sample was obtained, IL-1 was consistently elevated in one and consistently undetectable in the remaining 13. Interleukin-1 was undetectable in all eight healthy control subjects.

Mean plasma D-dimer and fibrinogen were significantly higher in crisis subjects than in steady-state subjects. D-dimer was 1062 ± 1043 (SD) ng/mL in crisis subjects and 656 ± 775 ng/mL in steady state

TABLE. TUMOR NECROSIS FACTOR, INTERLEUKIN-1, D-DIMER, AND FIBRINOGEN VALUES FOR 34 PATIENTS WITH SICKLE CELL DISEASE

Subject-Sample No. (Status)	TNF (pg/mL)	IL-1 (pg/mL)	D-dimer (ng/mL)	Fibr (mg/dL)	Occlusive Score	Active Complications*
2-4 (C)	<60	<75	185	—	1	None
2-5 (C)	<60	<75	280	—		
2-6 (C)	<60	<75	335	—		
6-2 (S)	<60	<75	1040	—	1	None
6-3 (S)	78	<75	235	—		
7-1 (S)†	175	<75	350	266	2	Retinopathy
9-1 (S)	60	<75	325	—	5	Aseptic necrosis
10-1 (S)	780	310	1040	—	3	None
11-1 (C)	72	<75	880	—	6	None
11-2 (C)	<60	<75	980	666		
11-3 (C)	64	<75	1200	608		
11-4 (C)	<60	<75	1425	670		
11-5 (S)	88	<75	1100	436		
12-1 (C)†	260	<75	300	272	3	Aseptic necrosis
12-2 (C)	240	<75	525	290		
12-3 (C)	305	<75	320	325		
12-4 (C)	330	<75	400	405		
12-5 (C)	340	<75	395	405		
12-6 (S)	205	<75	250	—		
13-1 (C)	68	<75	180	—	3	None
13-2 (C)	72	<75	205	338		
14-1 (C)	240	<75	320	205	1	None
14-2 (S)	380	<75	270	—		
15-1 (S)	290	<75	280	—	0	None
16-1 (S)	78	<75	160	234	0	None
17-1 (S)†	70	<75	210	358	2	None
18-1 (S)†	170	<75	100	—	0	None
19-1 (C)	60	<75	825	—	1	None
20-1 (C)	720	160	1900	637	3	None
21-1 (C)	<60	<75	295	195	1	None
21-2 (C)	<60	<75	328	282		
21-3 (S)	68	<75	1550	—		
22-1 (C)	<60	<75	3950	378	3	None
23-1 (S)	65	150	460	—	0	None
24-1 (S)	64	<75	170	—	1	None
25-1 (C)	60	<75	960	376	3	None
25-2 (C)	78	<75	935	—		
26-1 (S)	60	125	560	294	4	Aseptic necrosis
27-1 (C)	<60	<75	3375	771	1	None
28-1 (S)	<60	<75	325	533	3	Aseptic necrosis
28-2 (C)	<60	<75	500	—		

Abbreviations: TNF = tumor necrosis factor, Fibr = fibrinogen, C = crisis, and S = steady state.
*Other than crisis.
†Hb SC.

TABLE. TUMOR NECROSIS FACTOR, INTERLEUKIN-1, D-DIMER, AND FIBRINOGEN VALUES FOR 34 PATIENTS WITH SICKLE CELL DISEASE (CONTINUED)

Subject-Sample No. (Status)	TNF (pg/mL)	IL-1 (pg/mL)	D-dimer (ng/mL)	Fibr (mg/dL)	Occlusive Score	Active Complications*
29-1 (C)	340	<75	3200	—	1	None
29-2 (C)	340	<75	1975	—		
29-3 (C)	315	<75	3250	700		
30-1 (C)	<60	<75	305	224	3	None
30-2 (C)	135	<75	210	310		
30-3 (C)	210	<75	315	354		
31-1 (S)	<60	<75	250	265	1	None
32-1 (C)	75	160	2425	576	4	None
32-2 (C)	70	150	1975	—		
33-1 (C)	135	<75	2200	280	2	None
33-2 (C)	165	<75	300	—		
34-1 (S)	<60	<75	975	—	3	None
35-1 (S)	195	<75	3750	—	4	Retinopathy, leg ulcers
36-1 (C)	165	305	1475	365	3	None
37-1 (S)	240	<75	320	—	3	None
38-1 (C)	<60	<75	400	284	3	Leg ulcers
39-1 (C)	725	<75	260	719	1	None
39-2 (S)	245	<75	725	—		

Abbreviations: TNF = tumor necrosis factor, Fibr = fibrinogen, C = crisis, and S = steady state.
*Other than crisis.
†Hb SC.

subjects ($P<.01$). Fibrinogen was 425 ± 178 (SD) mg/dL in crisis subjects and 341 ± 101 mg/dL in steady-state subjects ($P<.05$). No correlation was observed between either TNF or IL-1 and D-dimer or fibrinogen.

No correlation was found between TNF or IL-1 and either the overall severity of vaso-occlusive disease as quantitated by the scoring system of Hebbel et al[13] or with the presence of specific vaso-occlusive complications (aseptic necrosis of the bone, leg ulcers, or proliferative retinopathy). There was also no correlation between TNF or IL-1 and either the current or usual level of pain as reported by each subject. Tumor necrosis factor was elevated in all of the samples from the four subjects with Hb SC, but the limited number of SC subjects studied prevents a determination of whether TNF is more frequently elevated in Hb SC disease than in Hb SS disease.

DISCUSSION

These results indicate that the majority of subjects with sickle cell disease have increased plasma levels of immunoreactive TNF, and some also have increased immunoreactive IL-1. Tumor necrosis factor levels varied among subjects but were fairly consistent for each individual subject. However, we do not have sufficient long-term serial data to determine whether TNF levels remain relatively constant over long periods of time in individual subjects.

Acute infections are known to be associated with elevated plasma cytokine levels,[11] and subjects with sickle cell disease are known to be unusually susceptible to infections.[1,2] Therefore, it is possible that occult or undiagnosed infections may have caused elevated cytokine levels in some of our subjects. However, we deliberately excluded any subject with a documented infection, and it is unlikely that occult infections were present in all of our subjects with elevated plasma cytokines. It is quite possible that reticuloendothelial dysfunction leading to impaired clearance of endotoxin absorbed from the gut may have caused elevated cytokines in some of our subjects,[12] but we did not

measure endotoxin levels. The relationship between elevated cytokines and abnormal immune function in sickle cell disease clearly deserves further investigation.

Among their many effects, TNF and IL-1 enhance endothelial adhesiveness,[7] activate white blood cells[7] and the coagulation cascade,[8,15] and cause elevations in acute-phase plasma proteins such as fibrinogen that are known to support erythrocyte adhesion to endothelium.[5,6,16,17] Each of these effects could contribute to the development of vascular occlusion in sickle cell disease,[3] but the lack of correlation between TNF or IL-1 levels and the clinical status of our subjects suggests that the contribution of elevated cytokines to vascular occlusion in sickle cell disease may be indirect or subtle. Also, we may have underestimated the true frequency of elevated cytokines in sickle cell disease because of the relative insensitivity of ELISA assays. Major physiologic effects are produced by concentrations of cytokines too low to be detected by ELISA.[8] Thus, further investigations of TNF and IL-1 in sickle cell disease using more sensitive bioassays are needed.

Literature Cited

1. Powars DR, Chaln LS, Schroeder WA. The variable expression of sickle cell disease is genetically determined. *Semin Hematol.* 1990;27:360-376.

2. Thomas AN, Pattison C, Serjeant GR. Causes of death in sickle-cell disease in Jamaica. *Br Med J.* 1982;285:663-667.

3. Francis RB Jr, Johnson CS. Vascular occlusion in sickle cell disease: current concepts and unanswered questions. *Blood.* 1991;77:1405-1410.

4. Stuart J, Johnson CS. Rheology of the sickle cell disorders. *Baillieres Clin Haematol.* 1987;1:747-760.

5. Dinarello CA. Interleukin-1 and the pathogenesis of the acute-phase response. *N Engl J Med.* 1984;311:1413-1418.

6. Beutler B, Cerami A. Cachectin: more than a tumor necrosis factor. *N Engl J Med.* 1987;316:379-384.

7. Movat HZ. Tumor necrosis factor and interleukin-1: role in acute inflammation and microvascular injury. *J Lab Clin Med.* 1987;110:668-681.

8. Bauer KA, ten Cate H, Barzegar S, Spriggs DR, Sherman ML, Rosenberg RD. Tumor necrosis factor infusions have a procoagulant effect on the hemostatic mechanism of humans. *Blood.* 1989;74:165-172.

9. Michie HR, Manogue KR, Spriggs DR, et al. Detection of circulating tumor necrosis factor after endotoxin administration. *N Engl J Med.* 1988;318:1481-1486.

10. van der Meer JWM, Endres S, Lonnemann G, et al. Concentrations of immunoreactive human tumor necrosis factor alpha produced by human mononuclear cells in vitro. *J Leukoc Biol.* 1988;43:216-223.

11. Girardin E, Grau GE, Dayer J-M, et al. Tumor necrosis factor and interleukin-1 in the serum of children with severe infectious purpura. *N Engl J Med.* 1988;319:397-400.

12. Thomson APJ, Dick M. Endotoxinaemia in sickle cell disease. *Clin Lab Haematol.* 1988;10:397-400.

13. Hebbel RP, Boogaerts MAB, Eaton JW, Steinberg MH. Erythrocyte adherence to endothelium in sickle-cell anemia: a possible determinant of disease severity. *N Engl J Med.* 1980;302:992-995.

14. Francis RB Jr. Elevated fibrin D-dimer fragment in sickle cell anemia: evidence for activation of coagulation during the steady state as well as in painful crisis. *Haemostasis.* 1989;19:105-111.

15. Bevilacqua MP, Pober JS, Majeau GR, Fiers W, Cotran RS, Gimbrone MA Jr. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. *Proc Natl Acad Sci USA.* 1986;83:4533-4539.

16. Perlmutter DH, Dinarello CA, Punsal PI, Colten HR. Cachectin/tumor necrosis factor regulates hepatic acute-phase gene expression. *J Clin Invest.* 1986;78:1349-1355.

17. Mohandas N, Evans E. Adherence of sickle erythrocytes to vascular endothelial cells: requirement for both cell membrane changes and plasma factors. *Blood.* 1984;64:282-287.